Exhibit C

Spatial and temporal control of gene therapy using ionizing radiation

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Activation of transcription of the *Egr-1* gene by X-rays is regulated by the promoter region of this gene. We linked the radiation-inducible promoter region of the *Egr-1* gene to the gene encoding the radiosensitizing and tumoricidal cytokine, tumour necrosis factor-α (TNF-α) and used a replication-deficient adenovirus to deliver the Egr-TNF construct to human tumours growing in nude mice. Combined treatment with Ad5.Egr-TNF and 5,000 cGy (rad) resulted in increased intratumoral TNF-α production and increased tumour control compared with treatment with Ad5.Egr-TNF alone or with radiation alone. The increase in tumour control was achieved without an increase in normal tissue damage when compared to tissue injury from radiation alone. Control of gene transcription by ionizing radiation *in vivo* represents a novel method of spatial and temporal regulation of gene-based medical treatments.

The potential of gene therapy in the treatment of cancer is the delivery of therapeutic agents to tumour cells to alter the malignant phenotype or to induce tumour cell cytotoxicity[1,2]. Gene therapy strategies currently under clinical investigation for the treatment of cancer include the study of genes that activate or encode cytotoxins and immune modulators[3,4]. These strategies are frequently limited by inherent drug resistance of tumour cells or by poor diffusion of the gene product. The cytotoxic proteins, such as ricin and pseudomonas endotoxin, must also be restricted to the local tumour site to avoid potential systemic toxicity. Methods used for localizing cytotoxic gene therapy for cancer include viral delivery systems that take advantage of tissue-specific receptors[5] and tissue-specific enhancers that limit transcription to certain cell types[6]. However, despite these advances, localizing gene therapy to the tumour remains a major obstacle. Furthermore, the temporal regulation of cytotoxic genes is not possible with constitutive promoters that have a high level of basal gene expression. Alternatively, transcriptional activation of a radiation-inducible promoter can be controlled by ionizing radiation within a specific volume and for a chosen period. We have taken advantage of both the killing effect and the targeting potential of ionizing radiation to achieve spatial and temporal regulation of TNF-α gene transcription and enhance tumour cell killing.

Tumour necrosis factor-α (TNF-α) is a cytokine that activates the cellular immune response[7] and is directly cytotoxic to some tumour cells[8]. Mechanisms of direct cell killing by TNF-α involve both apoptosis and necrosis[9,10]. When combined with radiation *in vivo*, TNF-α is reported to enhance tumour control through immune modulation[11]. We and others have reported that TNF-α enhances direct tumour cell killing *in vivo* and *in vitro* following exposure to ionizing radiation[12-17] and a clinical study that combined systemically (intravenously) administered TNF-α and therapeutic local/regional radiation demonstrated promising results in local tumour control[18]. In that study, increased serum concentrations of TNF-α correlated with an increase in local control in the irradiated tumour bed. However, systemic toxicity attributable to TNF-α limited the therapeutic efficacy of this treatment regimen. We propose that a gene therapy approach combining high intratumoral TNF-α levels induced by regional radiation exposure might limit systemic toxicity while achieving local tumour control.

Although numerous radiation-inducible genes[19-21], and proteins[22] have been identified, relatively few radiation inducible promoters/enhancers have been characterized[23,24]. Promoter/enhancers are DNA sequences that bind proteins which control gene transcription. DNA sequences that activate transcription after X-irradiation include AP-1 (ref. 23), the NFkB binding sequence[25] and the CC(A+T rich)$_6$GG (CArG) elements within the 5′ untranslated region of the early growth response (Egr-1) promoter[24]. We selected the CArG elements of the *Egr-1* promoter (425 bp upstream from the transcription start site) to regulate TNF-α, because these elements are inducible in several types of human tumour cells[24,26] (D.E.H., unpublished observation). We ligated a region containing the six CArG elements of the promoter/enhancer region of the *Egr-1* gene upstream to a *TNF-α* cDNA. The replication-deficient adenovirus type 5 (Ad5)[27,28] was used to deliver the Egr-TNF genetic construct to tumours. The Ad5.Egr-TNF vector was injected into the human epithelial tumour (SQ-20B) xenograft. SQ-20B is a radioresistant tumour cell

line derived from a human laryngeal carcinoma[29,30]. The schedule of delivery of Ad5.Egr-TNF and radiation was chosen to simulate treatment in the clinical setting. We show that the decrease in tumour volume after administration of Ad5.Egr-TNF and radiation is associated with TNF-α induction and an increase in apoptosis, as well as in necrosis and inflammation. These effects were not observed using the control Ad5(null) virus combined with radiation.

Ad5.Egr-TNF and therapeutic radiation

To study a potential interaction between Ad5.Egr-TNF and radiation, SQ-20B xenografts were grown to a mean volume of 160 mm^3 and injected with Ad5.Egr-TNF. Control tumours were uninjected (Fig. 1*a*). Ad5.Egr-TNF alone or radiation alone produced tumour regression to a mean of 30% (day 28) or 51% (day 25) of the original volume, respectively (Fig. 1*a*). Tumour regression in these groups of animals was followed by tumour regrowth to the original volume by day 42 and 38 post-treatment (Fig. 1*a*). In the group of mice treated with Ad5.Egr-TNF plus radiation, the mean tumour volume was reduced to 16% of the original volume at day 38 ($P < 0.05$) with no tumour regrowth. At day 60, Ad5.Egr-TNF combined with X-irradiation produced a 90% reduction in tumour volume in 12 of 16 tumours, compared with radiation alone (10 of 23 tumours), or Ad5.Egr-TNF alone (5 of 16 tumours; $P < 0.04$, ANOVA).

Tumours were treated with the Ad5(null) virus with and without radiation. Xenografts were grown to a mean volume of 152 mm^3. Tumours were injected with Ad5(null) virus twice weekly for 2 weeks, either alone or in combination with X-irradiation (5 Gy day^{-1}, 4 days per week) to a total dose of 40 Gy. Uninjected tumours were treated with a total dose of 40 Gy. We detected no effect on tumour growth of the Ad5(null) virus (1×10^8), with or without radiation (Fig. 1*b*). Similar results were obtained from tumours treated with Ad5(null) at 2×10^8 (data not shown). The data are consistent with our apoptosis findings *in vitro* and pathological studies, which demonstrate no interaction between null virus and radiation (see below).

Radiation cures a greater percentage of small tumours than of large tumours, which have a greater cell burden. In order to determine whether combined treatment with Ad5.Egr-TNF and radiation would be effective in controlling larger tumours, we divided tumours into 'small' and 'large' groups. The mean tumour volume of 160 mm^3 was chosen to divide tumours by size. The mean volume of the large tumours was 234 ± 25 mm^3 whereas the mean volume of the small tumours was 114 ± 6 mm^3 (large versus small tumours, $P < 0.001$). In the combined treatment group, the mean volume of large tumours at day 0 was 218 ± 14 mm^3, and the mean volume of small tumours was 96 ± 12 mm^3 ($P < 0.001$). After X-ray treatment alone, both small (<160 mm^3) and large (>160 mm^3) tumours initially regressed. However, regrowth to initial volume occurred in the large tumours, whereas small tumours did not regrow (Fig. 2*a*). The combination of radiation and Ad5.Egr-TNF produced a similar volumetric reduction of both large and small tumours without tumour regrowth (Fig. 2*b*). These data suggest that induction of the radiosensitizing cytokine TNF-α enhances the efficacy of radiation therapy in large tumours, which cannot be controlled effectively with radiation alone.

Radiation inducibility of Egr-TNF

To study radiation induction of TNF-α, SQ-20B xenografts were grown to a mean volume of 148 mm^3 in both hind limbs of nude

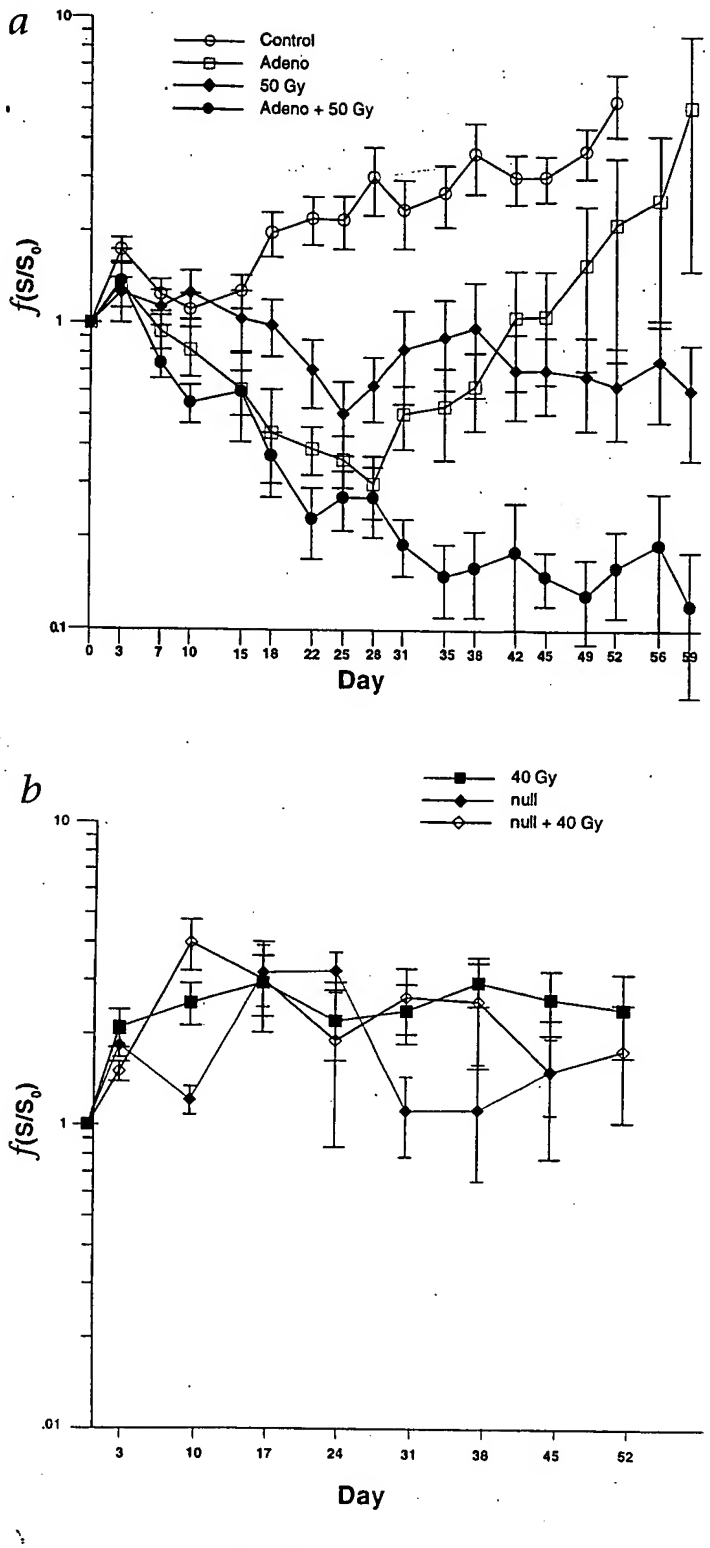

Fig. 1 Reduction of SQ-20B tumour volumes following combined treatment. SQ-20B xenografts were injected with $1x10^8$ PFU of Ad5(null virus) or 2×10^8 PFU of Ad.Egr-TNF (twice a week for two weeks). Control tumours were not injected. Tumours were irradiated (5 Gy per day, 4 days per week) to a total dose of 40 or 50 Gy. *a*, Volumes of xenografts are shown following treatment with: Control (untreated); radiation alone at 50 Gy; Ad.Egr-TNF alone; Ad.Egr-TNF and 50 Gy. Data are calculated as the percent of original (day 0) tumour volume and graphed as fractional tumour volume ± s.e.m. *b*, Volumes of xenografts are shown following treatment with: 40 Gy; Ad5(null virus); Ad5 (null virus) + 40 Gy.

mice. Each tumour was injected with 2×10^8 PFU Ad5.Egr-TNF. Tumours in the right hind limbs were irradiated with 5 Gy, four times each week, to a total of 50 Gy. Unirradiated tumours in the left limbs of the same mice served as controls. TNF-α concentrations in tumour extracts were analysed using an ELISA assay. Median TNF-α concentrations in irradiated tumours were 2,846, 4,300 and 20,000 pg mg^{-1} tumour protein at 7, 14 and 21 days, respectively. Unirradiated tumours had median TNF-α concentrations of 945, 950 and 2,643 pg mg^{-1}, respectively ($P = 0.058$, Kruskal-Wallis test)[34]. This ELISA does not detect murine TNF-α and is specific for TNF-α produced by the human cDNA (D.E.H., unpublished observations). These data demonstrate that a single administration of the Ad5.Egr-TNF vector is sufficient for radiation induction of TNF-α protein for at least 21 days, and that TNF-α levels accumulate when repeated induction is achieved during fractionated radiotherapy.

TNF-α immunohistochemistry

To analyse the distribution of Ad5.Egr-TNF, SQ-20B tumour xenografts were grown to a mean volume of 148 mm^3 in the hind limbs of nude mice. Tumours received a single injection of 2×10^8 PFU of Ad5.Egr-TNF and were irradiated with a single 5 Gy dose. Immunohistochemical staining of tumour cryosections with goat anti-human recombinant TNF-α antibody was performed 24 hours after irradiation. Granular intracytoplasmic staining for TNF-α was found within infected tumour cells, suggesting that TNF-α was being packaged for secretion (Fig. 3*a*). TNF-α was released by the infected cells and diffused into the tumour interstitium at 48 hours, as indicated by pink staining on non-infected tumour cells (Fig. 3*b*). The finding that TNF-α diffuses to adjacent tumour cells suggests that anti-tumour activity mediated by TNF-α when combined with radiation may be due to a bystander effect. Such an effect, produced by TNF-α gene therapy, is consistent with our recent finding demonstrating that a cell-based delivery system containing Egr-TNF-α achieves interactive killing with radiation in SQ-20B tumour xenografts even though the xenograft SQ-20B cells themselves did not contain the TNF-α gene[14].

Apoptosis induced in SQ-20B cells

To determine whether apoptosis contributes to tumour cell killing with the combination of radiation and TNF-α, we infected SQ-20B cells with Ad5.Egr-TNF at a multiplicity of infection (MOI) of 10, followed 24 hours later by a single dose of 20 Gy. Cells were fixed and DNA fragmentation was quantified by terminal transferase assay[31] and fluoresence-activated cell sorting analysis. Forty-eight hours after irradiation, cells treated with either 20 Gy X-irradiation alone (Fig. 4*a*) or Ad5.Egr-TNF alone (Fig. 4*b*) demonstrated no apoptosis. In contrast, 30% of the cells receiving combined treatment of Ad5.Egr-TNF and 20 Gy demonstrated DNA fragmentation (Fig. 4*c*) and membrane blebbing (not shown), both characteristics of apoptosis. The correlation between TNF-α concentration and apoptosis was verified by addition of 100 pg ml^{-1} human recombinant TNF-α to the culture medium 4 hours before a single dose of 20 Gy. This concentration of exogenous TNF-α produced a similar degree of apoptosis as compared with that achieved with a MOI of 10 (data not shown). Conversely, the addition of the Ad5(null) virus at a MOI of 10, followed by radiation, did not produce apoptosis. These results suggest that the combination of high levels of TNF-α and radiation results in tumour cell killing by apoptosis.

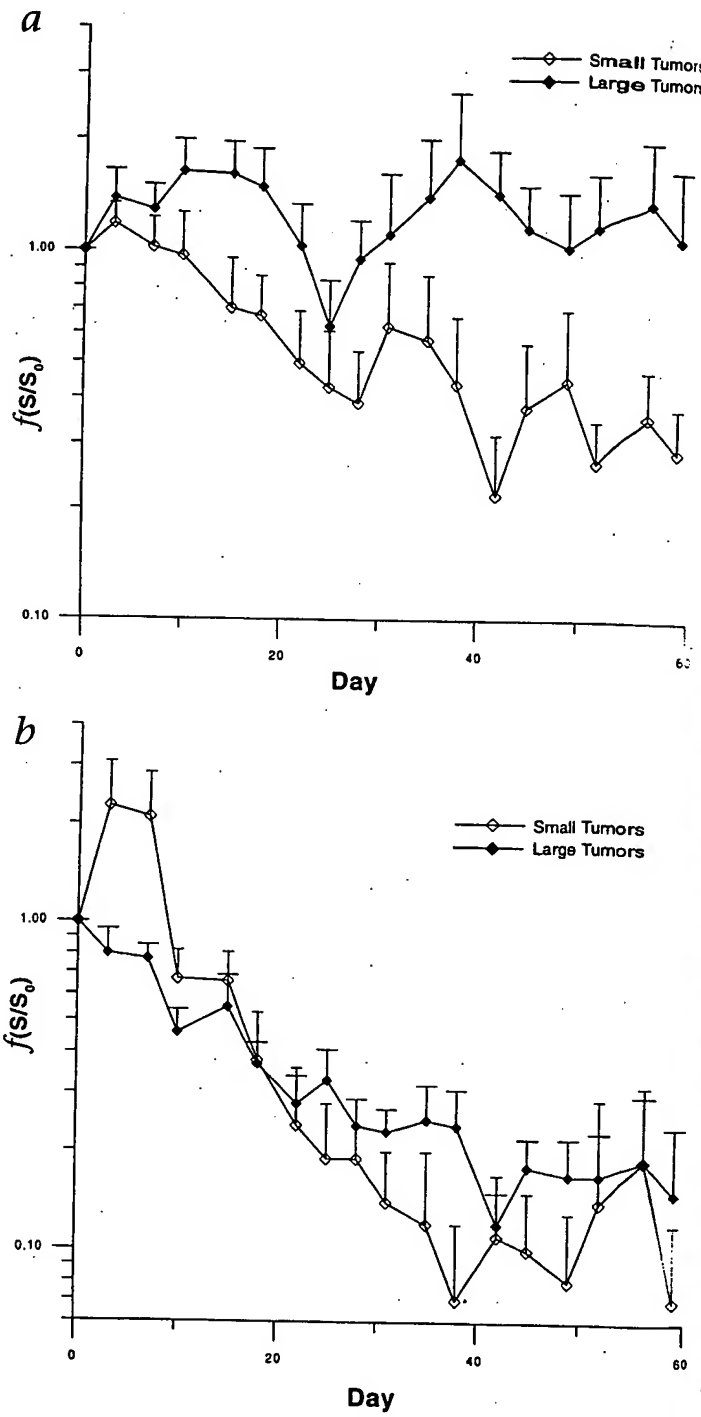

Fig. 2 Treatment effects on large versus small tumours. *a*, Mean volumes of large (>160 mm^3) and small (<160 mm^3) xenografts after treatment with radiation alone. The mean of large tumours at day 0 was 234 ± 25 mm^3 and the mean of small tumours at day 0 was 114 ± 6 mm^3. *b*, Mean volumes of large (>160 mm^3) and small (<160 mm^3) xenografts after treatment with Ad.Egr-TNF and radiation. The mean of large tumours at day 0 was 218 ± 14 mm^3 and the mean of small tumours was 96 ± 12 mm^3.

Necrosis in SQ-20B xenografts

To determine the extent to which necrosis is a component of tumour control, we analysed histologic sections of SQ-20B xenografts treated with two injections of Ad5(null) virus plus 20 Gy (5 Gy per day for 4 days) or with two injections of Ad.Egr-TNF

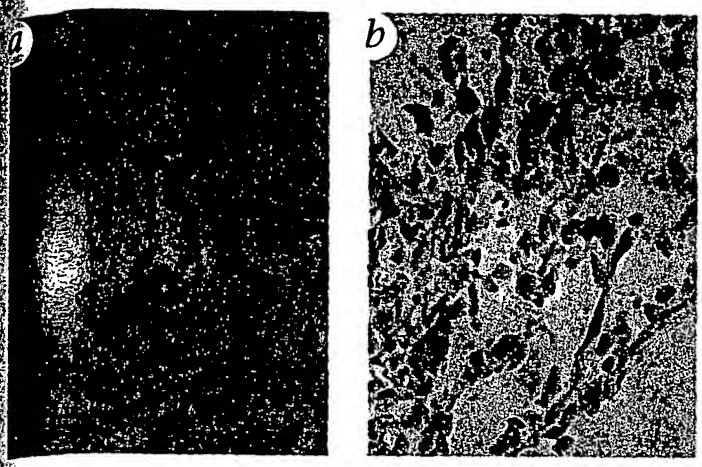

Fig. 3 TNF-α immunohistochemistry. Cryosections of the neoplasms were performed 24 hours after injection of 2×10^8 PFU Ad.Egr-TNF and 5 Gy X-irradiation. Sections were fixed in formalin and incubated with goat anti-human TNF-α monoclonal antibody followed by secondary antibody (horseradish peroxidase-conjugated rabbit anti-goat IgG) and stained for the presence of TNF-α protein. Slides were counterstained with haematoxylin. Shown are tumours stained at 24 hours after infection demonstrating TNF staining within granules (at ×1020 magnification) (*a*) and tumours excised at 48 hours after treatment (*b*) showing TNF in the tumour interstitium (×472 magnification).

plus 20 Gy. Xenografts treated with both Ad.Egr-TNF and radiation (Fig. 5*a*) had necrosis over a mean of 38 ± 8.3% of the low-power fields (ten fields counted) within seven days, whereas tumours treated with Ad5(null virus) and radiation had necrosis over a mean of 1.8 ± 0.97% of the low-power fields ($P = 0.007$) (Fig. 5*b*). This is supported by recent findings that direct tumour cell killing by TNF-α is associated with necrosis[7,9].

Toxicity of Egr-TNF combined with radiation

TNF-α mediated toxicity was evaluated in mice receiving 50 Gy and Ad.Egr-TNF. Serum levels of TNF-α were quantified by ELISA. No human TNF-α protein could be detected (<8 pg ml^{-1}) and there was no weight difference between animals treated with Ad5.Egr-TNF and X-irradiation and those receiving either treatment alone. No animals died in any of the treatment groups throughout the course of the experiment. In spite of mild local oedema and fibrosis, no loss of hind-limb mobility and no skin desquamation were observed in groups of animals receiving radiation and Ad.Egr-TNF. The soft tissue toxicity of Ad.Egr-TNF and radiation was comparable to that observed in animals receiving radiation alone. The minimal local effects of the TNF-α/radiation combination are similar to the effects observed in a clinical trial combining systemic recombinant TNF-α and radiotherapy[18]. The present data suggest that TNF-α production is localized to the tumour bed resulting in no systemic and only minimal local toxicity. Studies of possible long-term effects of TNF-α/radiation compared with radiation alone are under way.

Discussion

We have used TNF-α as a therapeutic gene for radiation-regulated gene therapy because of its direct anti-tumour effect and its interactive killing when combined with ionizing radiation. We observed that production of TNF-α in human tumour xenografts infected with the Ad.Egr-TNF and treated with radiation enhances

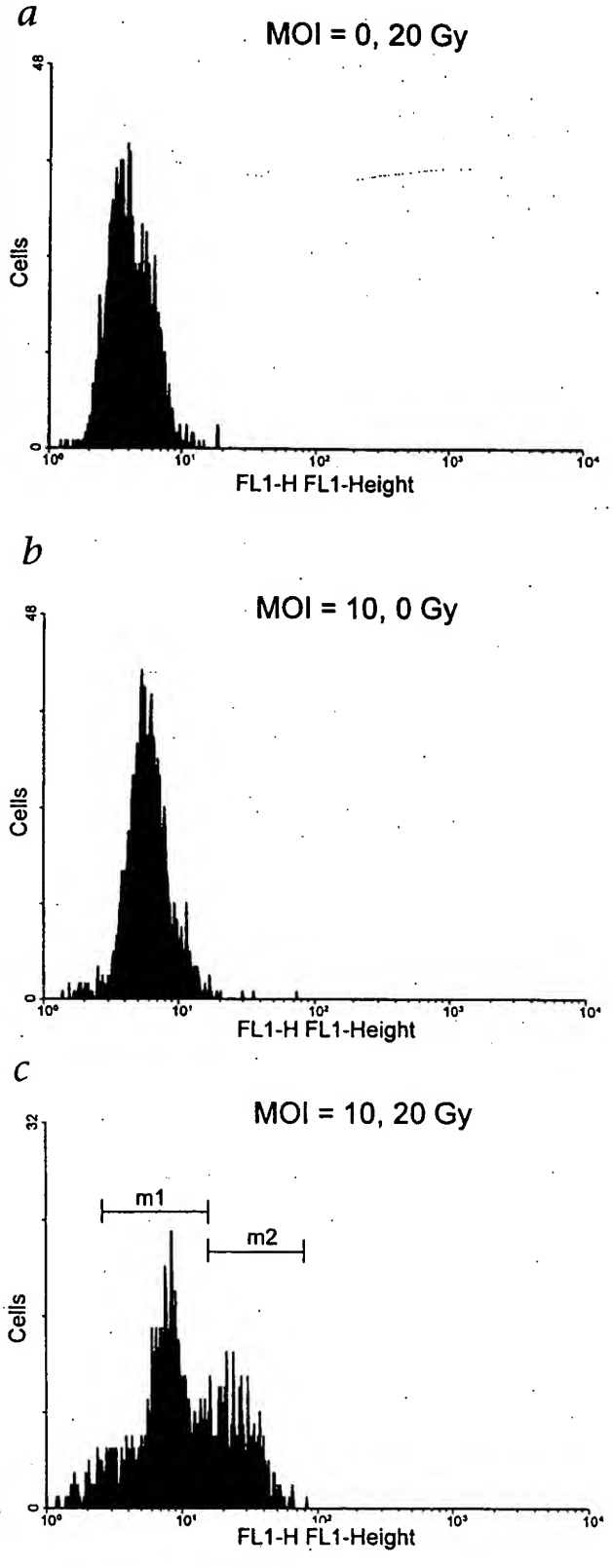

Fig. 4 Apoptosis of SQ-20B cells *in vitro*. SQ-20B tumour cells were treated with Ad.Egr-TNF at an MOI of 10, followed by 20 Gy, 24 hours later. At 48 hours after irradiation the presence of DNA strand breaks was evaluated. Analysed regions (gated regions) were set to quantify the number of cells undergoing DNA fragmentation. *a*, Cells treated with 20 Gy alone. *b*, Cells treated with Ad.Egr-TNF alone (MOI 10). *c*, Cells treated with 20 Gy and Ad.Egr-TNF (MOI 10).

tumour control as compared with radiation alone. These data are supported by *in vitro* studies demonstrating that TNF-α enhances tumour cell killing by radiation[13] and *in vivo* studies showing improved tumour control[11,16,17]. Unfortunately, a clinical trial combining TNF-α and radiation was limited by systemic toxicity[18]. The present work suggests that localized production of TNF-α may enhance tumour killing while avoiding systemic toxicity.

Interactive killing between TNF-α and radiation was associated with both apoptosis and necrosis. Although apoptosis has been associated with direct tumour cell killing by TNF-α (ref. 9) and radiation[32], we found that neither Ad.Egr-TNF, nor radiation alone resulted in apoptosis in the SQ-20B tumour cell line, whereas only cells receiving both TNF-α and radiation demonstrated both DNA fragmentation and membrane blebbing, characteristics of apoptosis. The apoptosis that we observed in this study was reproduced by treating SQ-20B tumour cells *in vitro* with recombinant TNF-α and radiation, but not by the combination of null virus (Ad5) and radiation, confirming that the effect was due to TNF-α rather than the vector itself. The apoptosis observed with concomitant Ad.Egr-TNF and radiation may have clinical significance, because Meyn *et al.* showed that murine tumours exhibiting a large apoptotic fraction following irradiation were more likely to be brought under control[32].

The inflammatory infiltrate seen in Fig. 5*a* suggests a further possible contribution of immune stimulation by TNF-α. The inflammatory component is consistent with findings observed with recombinant TNF-α combined with radiation *in vivo*[11]. Alternatively, the infiltrate may be due to the presence of the Ad5 vector itself, as this vector has been shown to induce IL-8 expression, which is a potent inducer of neutrophil infiltration[33]. However, SQ-20B xenografts injected with Ad5(null) virus demonstrated 36% less infiltration by neutrophils than tumours treated with Ad.Egr-TNF, suggesting that the inflammatory component observed in tumours treated with Ad.Egr-TNF was due to the presence of TNF-α.

Gene activation targeted by ionizing radiation is a new concept for cancer treatment whereby transcription of therapeutic genes is localized and regulated by ionizing radiation. The combination of proteins produced by targeted genes with the cytotoxic effect of ionizing radiation may enhance tumour cures without a significant increase in local or systemic toxicity. We found that radiation-induction of the Egr-TNF construct resulted in a ten-fold increase in TNF-α protein levels. The induction of TNF-α protein in irradiated tumours, compared with controls at 21 days, is consistent with our *in vitro* findings demonstrating an increase in *Egr-1* expression after irradiation[24]. Temporally fractionated radiation provides a method for repeated gene induction resulting in prolonged, accentuated gene expression. The concept of precisely activating gene transcription spatially and temporally by use of ionizing radiation may have wider applications in gene therapy and cancer treatment.

Methods

Growth of human tumour xenografts *in vivo*. We injected 10^6 SQ-20B tumour cells into the right hind limbs of nude mice. Xenografts were grown for 2–3 weeks, after which time tumours underwent spontaneous regression in less than 8% of mice. During treatment, tumour volumes were measured with calipers twice weekly and presented as per cent of original tumour volume. Tumours were grown to a mean volume of 152 mm^3 or 160 mm^3. Xenografts were injected with 1×10^8 to 2×10^8 PFU of Ad5(null virus) or 2×10^8 PFU of Ad.Egr-TNF (2 per week for 2 weeks). Control tumours were not injected. Irradiated mice were immobilized in lucite chambers and the entire body was shielded with lead except for the tumour bearing hind limb[14]. Tumours were irradiated (5 Gy day^{-1}, 4 days per week) to a total dose of 40 or 50 Gy using a Maxitron generator (1.88 Gy min^{-1}). Tumour volumes were calculated by the formula ($a \times b \times c/2$) which was derived from the formula for an ellipsoid ($\pi d^3/6$). Both the Ad5(null virus) and the Ad.Egr-TNF vector (GenVec, Rockville, Maryland) were recombined with a replication-deficient adenovirus type 5 (ref. 27). Tumours were treated with radiation alone; Ad5(null virus) alone; Ad.Egr-TNF alone; Ad5(null virus) combined with radiation; or Ad.Egr-TNF combined with radiation. Data were calculated as the percent of original (day 0) tumour volume and graphed as fractional tumour volume ± s.e.m. The regression rate of large (>160 mm^3) xenografts was compared with small (<160 mm^3) xenografts following treatment with radiation alone or Ad.Egr-TNF plus radiation. This tumour volume was selected because it represents the mean volume of the tumours treated with TNF-α.

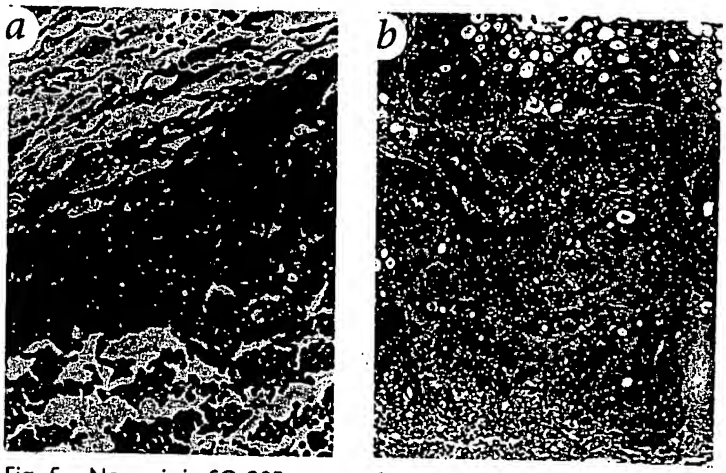

Fig. 5 Necrosis in SQ-20B xenografts receiving Ad.Egr-TNF and radiation. *a*, Tumours treated with Ad.Egr-TNF and X-irradiation (5 Gy per day for 4 days) were excised on day 7, embedded in paraffin, sectioned and stained with haematoxylin and eosin. *b*, Histologic sections of tumour treated with Ad5(null) virus and radiation (5 Gy per day for 4 days). Methods are as described as in the Methods section.

TNF-α expression in xenografts. SQ-20B xenografts were grown to a mean volume of 148 mm^3 in both hind limbs of nude mice. Each tumour was injected once with 2×10^8 PFU Ad.Egr-TNF 4 hours beforethe first dose of radiation. Tumours in the right hind limbs were irradiated with 5 Gy, 4 times each week to a total of 50 Gy. Unirradiated tumours in the left limbs of the same mice served as controls. Tumours were excised, placed in tubes and frozen in liquid nitrogen. Tumours were then homogenized in 500 µl sodium chloride/Tris buffer, pH 7.5, containing EDTA, dithiothreitol, and protease inhibitors. Homogenization on ice was for 30 s using a Brinkman Polytron and was followed by four cycles of freezing/thawing. Samples were centrifuged for 5 min at 10,000*g* and the supernatant assayed for TNF-α using a Quantikine TNF-α ELISA kit (R&D Systems, Minneapolis, Minnesota). Protein content was determined using the Bio-Rad Protein Micro Assay (BioRad, Melville, New York). The Kruskal-Wallis test was used for statistical analysis because it was determined that the data were not distributed normally[34].

TNF-α immunohistochemistry. Tumours were excised and frozen 24 hours after a single injection of 2×10^8 PFU Ad.Egr-TNF and 5 Gy X-irradiation. Cryosections of the neoplasms were performed and

tissue was fixed in formalin and incubated with goat anti-human TNF-α monoclonal antibody or normal goat serum at 1:200 dilution in PBS. Sections were then incubated with secondary antibody (horseradish peroxidase-conjugated rabbit anti-goat IgG) and stained for the presence of TNF-α protein, using Vector ABC reagent and diaminobenzidine (DAB) (Vector Labs, Burlingame, California). Slides were counterstained with haematoxylin and dehydrated, and coverslips were positioned before light microscopic evaluation.

Histology of SQ-20B xenografts. Tumours infected twice with Ad.Egr-TNF or Ad5(null virus) and treated with X-irradiation (5 Gy per day for 4 days), were excised on day 7 and fixed in 10% neutral buffered formalin. Tumours were then trimmed and processed in a Tissue Tek II Tissue Processor, embedded in paraffin, sectioned and stained with haematoxylin and eosin, and examined for signs of necrosis by light microscopy.

Terminal transferase quantification of apoptosis. SQ-20B tumour cells were treated with Ad.Egr-TNF at a MOI of 10, followed by 20 Gy 24 hours later. At 48 hours after irradiation, the presence of DNA breaks was evaluated by a modification of the nick-end translation method for *in situ* labelling of DNA breaks. Cells were fixed with 1% buffered methanol-free formaldehyde for 15 min on ice and stored in 70% ethanol overnight at –20 °C, rehydrated by washing twice in PBS and incubated in TDT buffer (25 mM Tris-HCl, 200 nM potassium cacodylate, 5 mM cobalt chloride at pH 6.6), 0.3 unit per μl terminal deoxynucleotidyl transferase and 3 nM biotin-15-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, Indiana) at 37 °C for 1 hour. The reaction was stopped by addition of ice-cold PBS and the cells were incubated for 30 min at room temperature in the dark in a solution containing 4× SSC buffer (Sigma), 5 μg ml^{-1} avidin-fluorescein-isothiocyanate (Boehringer Mannheim), 0.1% Triton X-100 (vol/vol), and 0.5% nonfat milk. The reaction mixture was washed once in ice-cold PBS containing 0.1% Triton X-100 (vol/vol), and cells were suspended at a concentration of 10^4 cells per ml in PBS containing propidium iodide (5 μg ml^{-1}) and 0.1% RNase (for 30 min at room temperature). The red (propidium iodide) and green (fluorescein) fluorescence was measured with a FACSCAN flow cytometer (Becton Dickinson, San Jose, California), and the data were analysed with LYSYS II software (Hewlett Packard, Palo Alto, California). Analysis regions (gated regions) were set to quantify the number of cells undergoing DNA fragmentation. Cells were treated with 20 Gy alone, Ad.Egr-TNF alone (MOI 10) or 20 Gy and Ad.Egr-TNF (MOI 10).

Acknowledgements

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